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INTERMEMBRANE TRANSFER OF BAND 3, THE ERYTHROCYTE ANION TRANSPORT PROTEIN, FROM SONICATED PHOSPHOLIPID VESICLES TO CELLS

Stephen Lloyd Cook

1983

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INTERMEMBRANE TRANSFER OF BAND 3,
THE ERYTHROCYTE ANION TRANSPORT PROTEIN,
FROM SONICATED PHOSPHOLIPID VESICLES TO CELLS

A DISSERTATION
SUBMITTED TO THE DEPARTMENT OF CELL BIOLOGY
AND THE SCHOOL OF MEDICINE
OF YALE UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF MEDICINE

By
Stephen Lloyd Cook
1983

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ACKNOWLEDGMENTS

I would like to thank:

Dr. Vincent T. Marchesi, for sponsoring this project.

Dr. Stanley R. Bouma, whose previous experiments laid the groundwork for this research.

Alexandra C. Newton, whose recent experiments have clarified my results.

James E. Ferrell, for "helpful discussions."

Dr. Wray H. Huestis, Stanford University Department of Chemistry, in whose laboratory this research was performed in the summer of 1980, for her optimism and guidance.

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LIST OF ABBREVIATIONS

AChE, acetylcholinesterase; ATP, adenosine triphosphate; 6-CF, 6-carboxyfluoroscein; Con A-Sepharose, Concanavalin A-Sepharose 4B; cmp, counts per minute; DIDS, diisothiocyanostilbene disulfonic acid; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; eosin MA, eosin maleimide; LML, large multilamellar liposome; LUV, large unilamellar vesicle; lyso-PC, lyso-phosphatidylcholine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PG, phosphatidyl-glycerol; PS, phosphatidylserine; SDS, sodium dodecylsulfate; SUV, small unilamellar vesicle.

ABSTRACT

When sonicated dimyristoylphosphatidylcholine (DMPC) vesicles are incubated with erythrocytes, several cell membrane proteins become associated with vesicles; these protein-vesicle complexes can be separated from the cells by centrifugation (Bouma, et al., 1977). One of the proteins extracted by vesicle treatment is band 3, the red cell anion transport protein. Vesicle-associated band 3 retains its native membrane orientation and its ability to transport anions. Purified band 3-vesicle complexes can be used to introduce band 3 into cells: if these vesicles are incubated with erythrocytes in which anion transport has been irreversibly inhibited, a significant percentage of the cells recover the ability to transport anions. Recent experiments have shown that the protein-vesicle complexes are produced by both cell membrane budding and by intermembrane protein transfer (Ott, et al., 1981; Huestis, personal communication). Reintroduction of band 3 into cells appears to occur by intermembrane protein transfer and not by cell-vesicle fusion.

INTRODUCTION

The past two decades have seen a remarkable increase in knowledge about cell membranes and their components, and an increasing sophistication in the methods employed to study them. The cell membrane is now known not to be just a passive barrier between the external environment and the cytoplasm, but an active participant in many aspects of cell physiology.

The model of membrane structure that is most widely accepted is the fluid mosaic model proposed by Singer and Nicolson (1972). According to this model, the basic structure of the membrane is a phospholipid bilayer modified by the presence of membrane-associated proteins (Figure 1). These proteins can be classified according to their mode of interaction with the bilayer. Intrinisic (or integral) proteins interact extensively with the hydrophobic acyl chain region of the bilayer. These proteins can be dissociated from the membrane only by methods that disrupt the integrity of the membrane bilayer (e.g., detergents). The other major class of proteins, extrinsic (or peripheral) proteins, bind to the surface of intrinsic proteins and do not interact with the bilayer. These proteins can be removed from the membrane by conditions that leave the membrane bilayer intact (e.g., high ionic strength solutions). Transmembrane proteins, a type of intrinsic protein, completely traverse the bilayer and interact with both the external and cytoplasmic environments. In the fluid mosaic model, membrane proteins can undergo translational motion within the plane of the bilayer (unless restricted by interactions with other proteins), but rotation of proteins from one side of the membrane to the other does not occur.

One of the most powerful tools in the study of cell membranes has been the use of model systems composed of synthetic or natural phospholipids. When these lipids are dissolved in aqueous solutions, they spontaneously form stable aggregates known as large, multilamellar liposomes (LMLs) (Bangham, et al., 1965). These are spheres of lipid bilayers arranged concentrically like the layers of an onion, several thousand angstroms in diameter (Figure 2). When LMLs are subjected to ultrasonication, they become one-bilayer spheres with diameters of about 250 Å known as small, unilamellar vesicles (SUVs) (Huang, 1969) (Figure 3). Large, unilamellar vesicles (LUVs), up to 800 Å in diameter, can be prepared by injection methods that do not involve sonication (Kremer, et al., 1977; Gerritsen et al., 1979).

One of the physical properties of purified phospholipid dispersions is a phase transition that occurs when they are passed through a critical temperature range. This transition is accompanied by a decrease in bilayer thickness (Chapman, et al., 1967), an increase in the rotational motion of the acyl chains (Trauble, 1972), a decrease in the order parameter of electron spin resonance probes (Hubbell and McConnell, 1971), and a decrease in apparent microviscosity measured with fluorescent probes (Shinitzsky and Barenholz, 1978). The phase transition is thought to represent a change from a state in which the phospholipid acyl chains are highly ordered (the "solid" or "gel" phase) to a state in which the acyl chains are more randomly oriented and the phospholipids can undergo rapid diffusional motion within the bilayer plane (the "fluid" or "liquid-crystalline" phase) (Trauble, 1972).

Figure 1: The fluid mosaic model of membrane structure, a phospholipid bilayer with associated intrinsic (A), extrinsic (B), and transmembrane (C) proteins.

Figure 2: Part of a large, multilamellar liposome (LML).

Figure 3: A small, unilamellar vesicle (SUV).

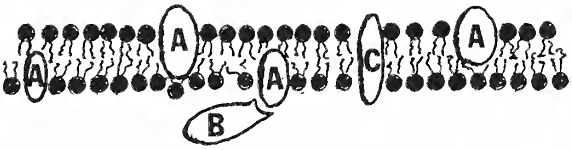


Figure 1.

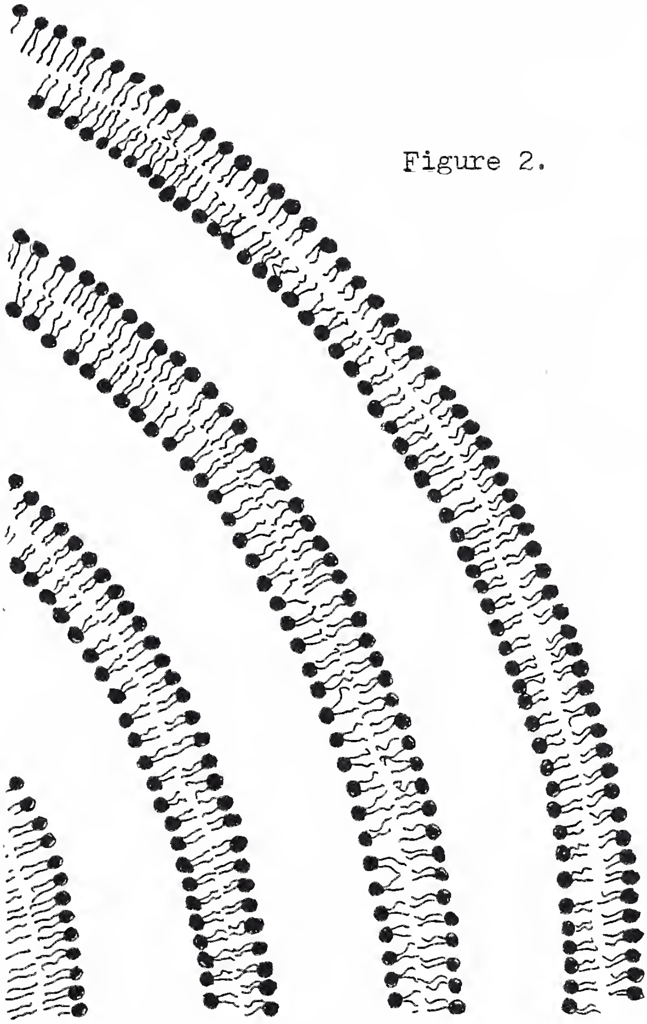


Figure 2.

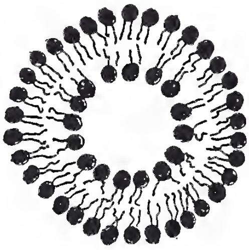


Figure 3.

LULs composed of pure phospholipids with identical acyl chains, such as dimyristoylphosphatidylcholine (DMPC), which has two saturated fourteen carbon chains, undergo a phase transition over a narrow (1-2^{o}C) temperature range. The phase transition range is broader for dispersions of more than one lipid (Shimshick and McConnell, 1973) and for SUVs (Lentz _et al_., 1976; Cook _et al_., 1980). At temperatures below the phase transition range, lipid bilayers are in the solid phase; at temperatures above the phase transition range, they are in the fluid phase. At temperatures within the range, both phases co-exist (Lee, 1977).

Another important physical property of lipid dispersions is surface charge. Dispersions of neutral phospholipids such as phosphatidylcholine (PC) and sphingomyelin have no net charge at physiological pH. Acidic phospholipids such as phosphatidylserine (PS) and phosphatidylglycerol (PG) confer net negative charge to liposomes and vesicles. Negatively charged dispersions can also be created by incorporating fatty acids or dicetylphosphate into neutral bilayers. No positively charged phospholipids occur naturally, but mixing stearylamine with neutral phospholipids generates positively charged dispersions.

It is these two physical characteristics, fluidity and surface charge, that determine the interactions of model membranes with each other and with cell membranes. Vesicles interact with cells in a variety of ways. These include:

1) Adsorption to the cell surface
2) Exchange of lipid components
3) Endocytosis of vesicles
4) Fusion

5) Induction of membrane budding

6) Intermembrane protein transfer

These interactions are illustrated schematically in Figures 4 through 9.

Adsorption (Figure 4) refers to vesicles adhering to cell surface proteins. The compositions of the cell and vesicle bilayers remain unchanged and their structural integrity is maintained. Pagano and Takeichi (1977) found that adsorption is the predominate interaction of cultured fibroblasts and solid phase PC vesicles. Most of the vesicles were released by digesting the cell surface proteins with trypsin.

Similar results have been obtained examining the interactions of positively charged DMPC/stearylamine vesicles with erythrocytes (Martin and MacDonald, 1976b; Cook and Huestis, unpublished results). At temperatures below their phase transition range, these vesicles adsorb to, and agglutinate the cells. When the temperature is raised above the vesicle phase transition range, the vesicles are released from the cells and are free to interact with the cell membrane in other ways. If the cells are pre-treated with neuraminidase, which cleaves negatively charged neuraminic acid residues from cell surface proteins, the vesicles do not agglutinate the cells regardless of the vesicle phase state.

Adsorbed vesicles appear to leak enclosed low molecular weight contents (Szoka, et al., 1979; Renswoude and Hoekstra, 1981). This process will be discussed in greater detail below.

Exchange of lipid components. When vesicles are incubated with cells, exchange of lipid occurs, producing the insertion of vesicle phospholipid into the cell membrane (Huang and Pagano, 1975) and vice versa (Bouma, 1980). Exchange of cholesterol between cells and vesicles has

also been described (Bruckdorfer, et al., 1969; Grunze and Deuticke, 1974). Lipid exchange occurs between cells and mixed phase vesicles (Grant and McConnell, 1973) and between cells and fluid vesicles (Huang and Pagano, 1975). Indirect evidence suggests that exchange also occurs with solid phase vesicles, but at a much slower rate (Cook et al., 1980).

Lipid exchange can be explained by two mechanisms. In one, lipid monomers diffuse through the aqueous medium between cells and vesicles without cell-vesicle contact occurring (Figure 5A). In the other mechanism, cells and vesicles transiently collide, and lipid transfer takes place through the point of contact between the bilayers (Figure 5B). It is unknown which of these two processes occurs (or which predominates if both occur). Thermodynamic considerations appear to favor a collisional model: the free energy for transfer of phospholipids from a non-polar to an aqueous environment--15.1 kcal/mole for dipalmitoylphosphatidylcholine (DPPC) (Smith and Tanford, 1972)--gives the diffusion model a much higher energy of activation. Studies examining exchange of lipid between DMPC and DPPC vesicles provided results that favor a diffusion model (Martin and MacDonald, 1976a). In these experiments, however, exchange occurred only with mixed phase vesicles. Lateral phase separations may lower the free energy for monomer formation. Because cell-vesicle lipid exchange occurs with all vesicle phase states, vesicle-vesicle exchange may not be an accurate model system for this process.

Endocytosis of vesicles (Figure 6) is the uptake of vesicles into the lysosomal apparatus of cells. This process requires metabolic energy and can be inhibited with cytochalasin B or inhibitors of glycolysis and

Figure 4: Adsorption of vesicles to cell surface proteins.

Figure 5: Lipid exchange between vesicles and cells: (A) Diffusion model, (B) Collision model.

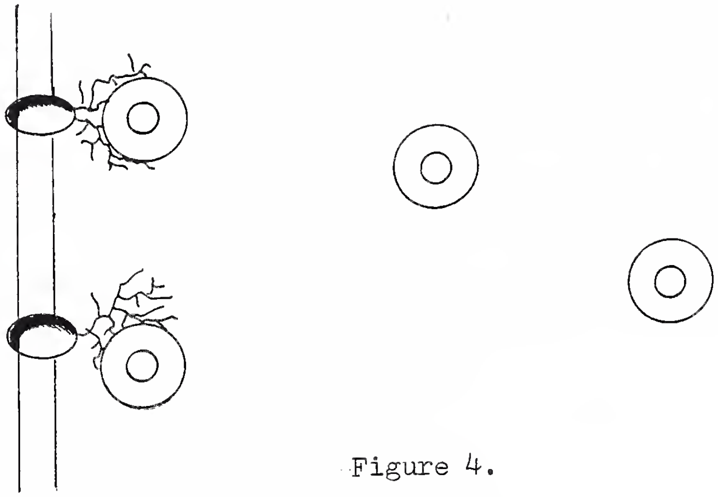


Figure 4.

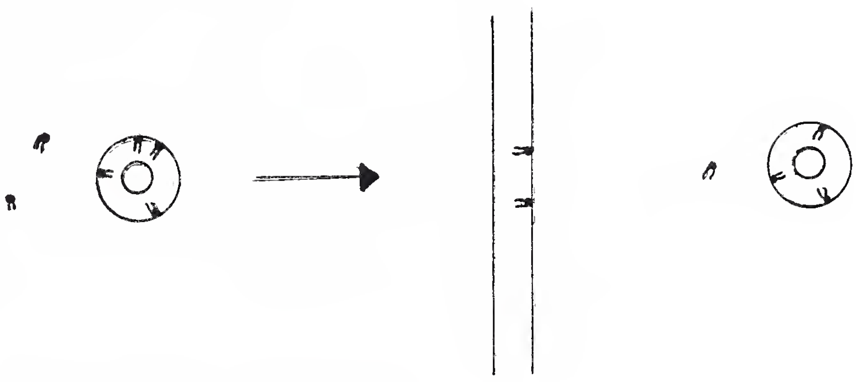


Figure 5A.

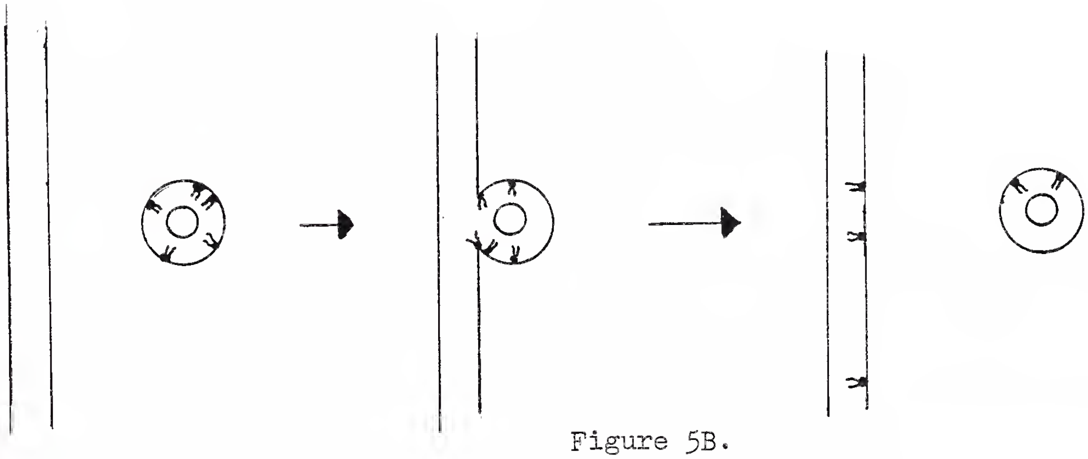


Figure 5B.

respiration used together. Endocytosis occurs regardless of the vesicle surface charge or fluidity (Poste and Papahadjopoulos, 1976).

Fusion (Figure 7) describes the insertion of the vesicle bilayer into the cell membrane accompanied by transfer of vesicle contents into the cytoplasm. Cell-vesicle fusion has attracted interest as a means of introducing drugs, nucleic acids, and other molecules into cells for experimental and clinical purposes (for several reviews, refer to Gregoriadis and Allison, 1980), and as a model for membrane-membrane fusion *in vivo*.

Cell-vesicle fusion is difficult to unequivocally demonstrate experimentally. In many systems intended to study fusion, other types of cell-vesicle interactions cannot be controlled for. For example, if vesicles whose enclosed space contains a label are incubated with cells and some of the label becomes associated with the cells, this result could be interpreted as indicating that cell-vesicle fusion had taken place. This result, however, could also be due to vesicle adsorption or endocytosis. Some of these problems have been surmounted by the development of two model systems for cell-vesicle fusion: vesicle-vesicle fusion and vesicle-induced cell-cell fusion. The reliability of these systems as models for cell-vesicle fusion is unclear. Nonetheless, from these studies and from the better-controlled attempts to demonstrate cell-vesicle fusion, a consistent picture of the conditions necessary for membrane-membrane fusion has emerged.

Weissman, *et al*. (1977) demonstrated that negatively charged PC/dicetylphosphate vesicles containing horseradish peroxidase could introduce this enzyme into three different cell lines. Delivery was increased

Figure 6: Endocytosis of a vesicle.

Figure 7: Fusion of a vesicle with a cell membrane.

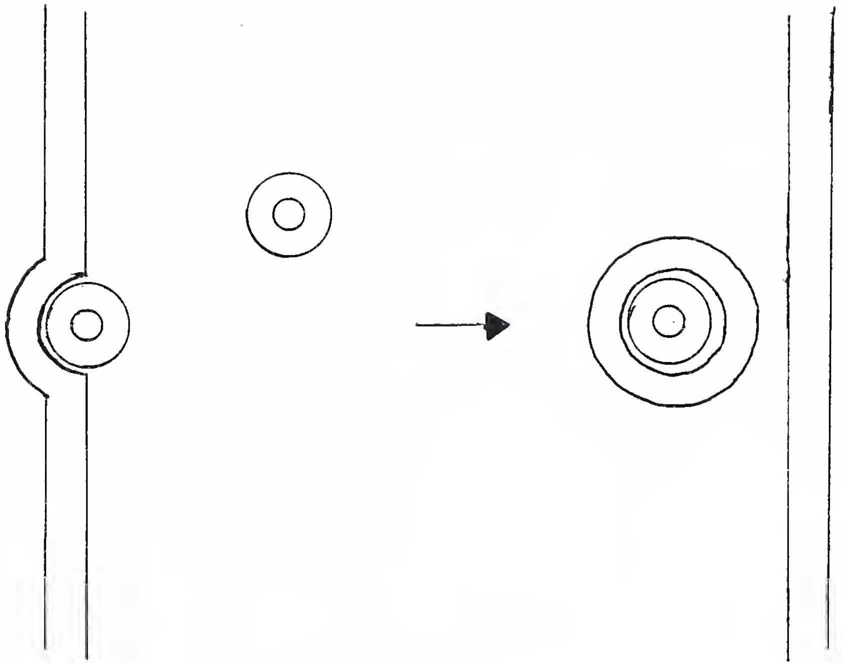


Figure 6.

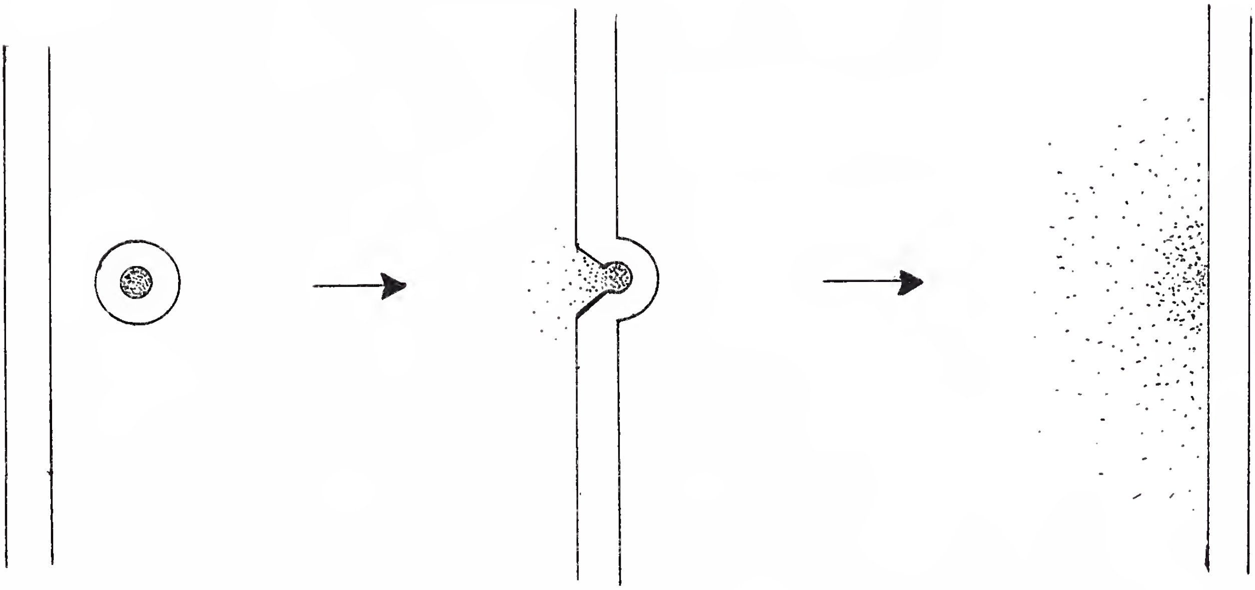


Figure 7.

two- to five-fold by the inclusion of lyso-phosphatidylcholine (lyso-PC) in the vesicles. Uptake from neutral PC vesicles was not observed. This process was not affected by inhibitors of endocytosis. Electron microscopy revealed that the location of the enzyme was cytoplasmic, rather than inside adsorbed vesicles.

Similar results were obtained by Poste and Papahadjopoulos (1976), who examined the transfer of enclosed sucrose from vesicles to cells. They found that fluid, negatively charged PS/PC vesicles can fuse with cell membranes, but neutral and solid phase vesicles (regardless of surface charge) cannot. Endocytosis and cellular uptake of leaked vesicle contents were controlled for in these experiments.

In contrast to these results, several reports have claimed to demonstrate cell-vesicle fusion using PC vesicles, but these studies have been criticized for methodological flaws. Pagano and Huang (1975) claimed to observe fusion with PC vesicles, but did not adequately control for endocytosis (Poste and Papahadjopoulos, 1976). Weinstein, et al. (1977) and Pagano and Takeichi (1977) studied the interaction of cells with PC vesicles whose enclosed volume contained the fluorophore 6-carboxyfluoroscein (6-CF). Due to self-quenching, probe molecules within the vesicles exhibited little fluorescence. When the vesicles were incubated with cells, cell-associated fluorescence was observed. It was concluded that this fluorescence resulted from cell-vesicle fusion and, as a result, spreading of the probe throughout the cytoplasm.

This interpretation was challenged by Szoka, et al. (1979), who observed that incubation of cells with vesicles containing 6-CF produces an efflux of probe from the vesicles. As noted above, this phenomenon

appears to be due to a vesicle permeability defect to small molecules that occurs when vesicles associate with cell surface proteins. The cell-associated fluorescence seen when vesicles containing 6-CF were incubated with cells could have been due to reduced self-quenching produced by lower probe concentrations within adsorbed vesicles, or to cell uptake of free probe released near the cell surface. It was concluded that unless efflux of the probe from the adsorbed vesicles could be controlled for, this system is unsuitable for studying cell-vesicle fusion.

Vesicle-vesicle fusion has been demonstrated with vesicles containing PS (Papahadjopoulos, et al., 1974; Miller and Racker, 1976), PG (Papahadjopoulos, et al., 1976b), fatty acids (Kantor and Prestegard, 1975), and brain gangliosides (Martin and MacDonald, 1976a), but not with pure PC vesicles. PC vesicles do form larger structures when incubated at temperatures within the phase transition range, but the mechanism of this process is intermembrane lipid transfer, not fusion (Papahadjopoulus, et al., 1976a; Martin and MacDonald, 1976a).

Several investigators have induced cell-cell fusion using vesicles. This process presumably involves fusion of a vesicle with the membranes of two cells. Papahadjopoulos et al. (1973) produced extensive cell-cell fusion with fluid PS and PS/PC vesicles. Neither PC vesicles nor solid phase vesicles induced fusion. Martin and MacDonald (1976c) observed some cell-cell fusion when incubated with vesicles containing lyso-PC or stearylamine, and extensive fusion with vesicles containing both. Under the conditions described, these vesicles were in a fluid phase state (Cook and Huestis, unpublished observations).

To summarize, the bulk of data obtained from experiments studying cell-vesicle fusion, vesicle-vesicle fusion, and vesicle-induced cell-cell fusion indicates that fluid vesicles with a positive or negative surface charge and/or containing lyso-PC can fuse with cell membranes, but neutral PC vesicles and solid phase vesicles cannot (Poste, 1980).

Induction of membrane budding and _intermembrane protein transfer_. These two processes will be discussed together since both happen simultaneously in the same experimental system. When DMPC vesicles are mixed with erythrocytes, several membrane proteins become associated with the vesicles. These protein-vesicle complexes can be separated from the cells by centrifugation (Huestis, 1976). If the protein-containing vesicles are analyzed by ultracentrifugation on sucrose gradients, two vesicle populations are observed (Huestis, personal communication). One of the populations contains vesicles whose lipid composition is similar to that of the red cells and whose enclosed volume contains hemoglobin (Ott, _et al_., 1981). The lipid composition of the second population is almost entirely DMPC. These vesicles contain no hemoglobin (Huestis, personal communication). Aside from hemoglobin, the protein composition of these two groups of vesicle-protein complexes is identical.

The first group of vesicles appears to be produced by budding from the cell membrane. Mixing fluid DMPC vesicles with red cells produces an almost immediate cell shape transformation from the normal discocyte conformation to an echinocyte (Ott, _et al_., 1981). With further incubation, the cells become spherocytes (Bouma, 1980). Scanning electron microscopy of the echinocytes reveals vesicles budding from the tips of the echinocytes (Ott, _et al_., 1981).

ATP depletion of erythrocytes also produces a discocyte-to-echinocyte shape change and the formation of membrane buds (Lutz, *et al.*, 1977; Sheetz and Singer, 1977). Vesicle treatment of red cells does not produce ATP depletion, so these two similar processes appear to occur by different mechanisms (Ott, *et al.*, 1981).

It has been proposed that vesicle-induced budding is the result of lipid transfer from the vesicles to the outer monolayer of the cell membrane (Ott, *et al.*, 1981). The resulting overabundance of lipid in the outer monolayer relative to the inner monolayer causes the membrane to "buckle" into an echinocyte conformation. In the tips of the echinocytes, two segments of the inner monolayer come into contact. The inner monolayer of the red cell contains all of the membrane PS (Verkleij, *et al.*, 1973). Therefore, echinocyte formation brings two PS-containing membranes into contact. These two membranes fuse, and the vesicle so formed dissociates from the cell. This mechanism is illustrated in Figure 8.

The second group of vesicle-protein complexes is created by a completely different process. Analysis of the lipid composition and enclosed contents of these vesicles reveals that they are the original DMPC vesicles that have acquired proteins from other membranes (Newton and Huestis, personal communication). This intermembrane protein transfer, just like lipid exchange (see above), could occur by either of two mechanisms: one is diffusion of proteins through the aqueous medium between the membranes; the other, through membrane-membrane collisions with transfer of proteins through the area of contact. The identities of several of the transferred proteins are known (see below). All are

Figure 8: Vesicle-induced membrane bud formation: treatment of erythrocytes with DMPC vesicles changes the cells from normal discocytes (A) to echinocytes (B); in the tips of the echinocytes (C), the inner monolayers fuse, creating a vesicle-bud that dissociates from the cell membrane (D).

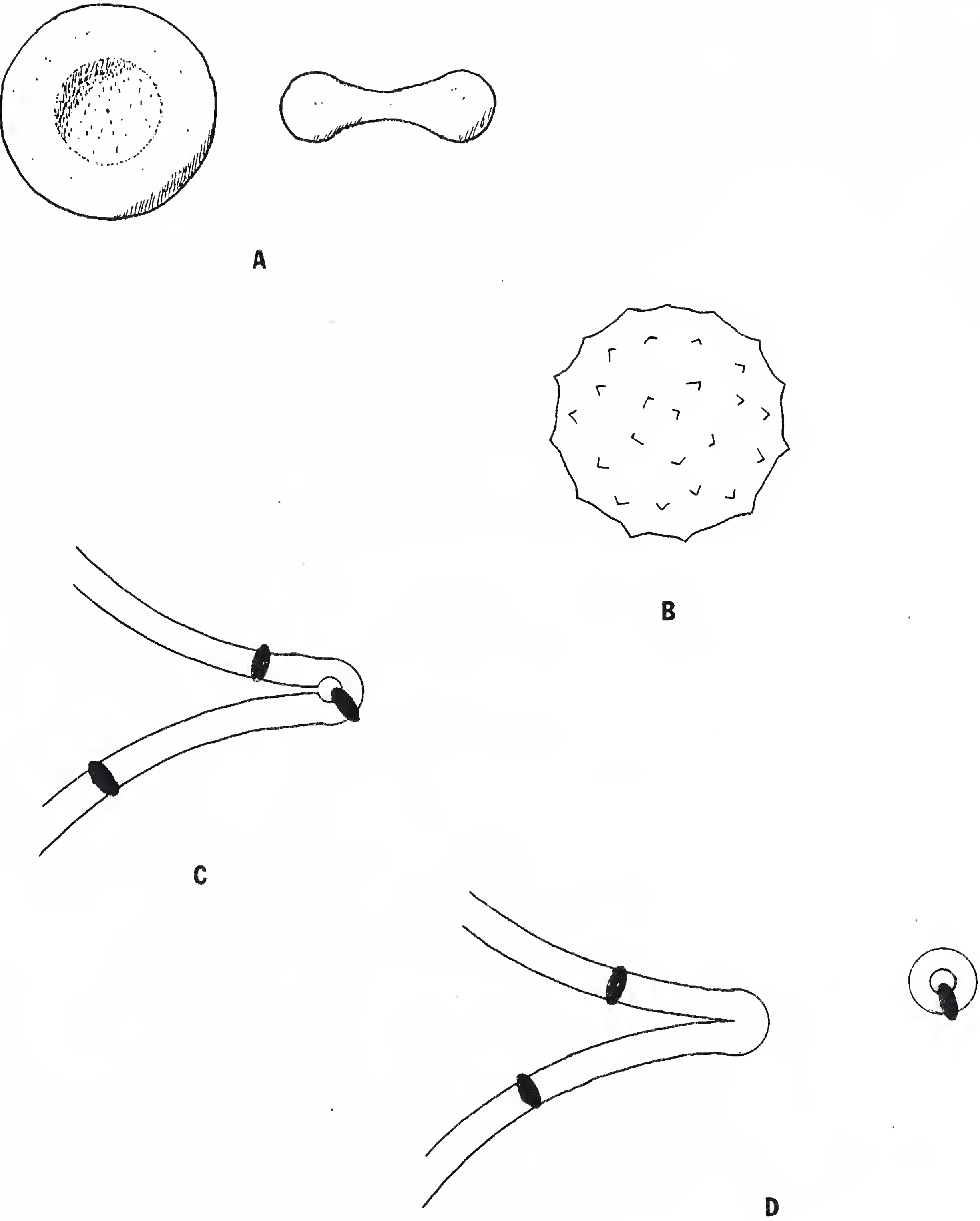


Figure 8.

intrinsic proteins. Several are transmembrane proteins. Therefore, it is extremely unlikely that these proteins would spontaneously dissociate from a lipid bilayer, and transfer of these proteins probably occurs through membrane-membrane collisions.

The "transfer vesicles" appear to acquire proteins from both the "vesicle-buds" and the cell membrane. If DMPC vesicles are mixed with vesicle-buds, intervesicle protein transfer occurs, and a population of transfer vesicles is formed. Likewise, incubation of DMPC vesicles with cells that have completed the discocyte-to-echinocyte shape transformation--and have thus completed vesicle budding--also produces transfer vesicles (Huestis, personal communication). The percentage of transfer vesicles that acquire proteins from cells versus the percentage that acquire proteins from vesicle-buds in unknown. Protein transfer between cells and vesicles and between vesicles and other vesicles is shown in Figure 9.

Several of the cell proteins that are "extracted" into vesicle-buds and transfer vesicles have been identified. One is the red cell acetylcholinesterase (AChE), an intrinsic protein (Ott, *et al.*, 1975) that can be removed with greater than 90% efficiency using fluid DMPC vesicles. Extracted AChE retains its native orientation and function in the vesicle membrane (Bouma, *et al.*, 1977). Glycophorin, a glycosylated transmembrane protein (Marchesi, 1979), is also removed (Ott, *et al.*, 1981). One extracted protein appears to be associated with a red cell sodium channel similar to ion channels found in nerve and muscle cells (Huestis, 1977). Another migrates on SDS-polyacrylamide gels with an apparent

Figure 9: Intermembrane protein transfer of an intrinsic protein.

(A) Protein transfer between vesicles

(B) Protein transfer from a cell to a vesicle

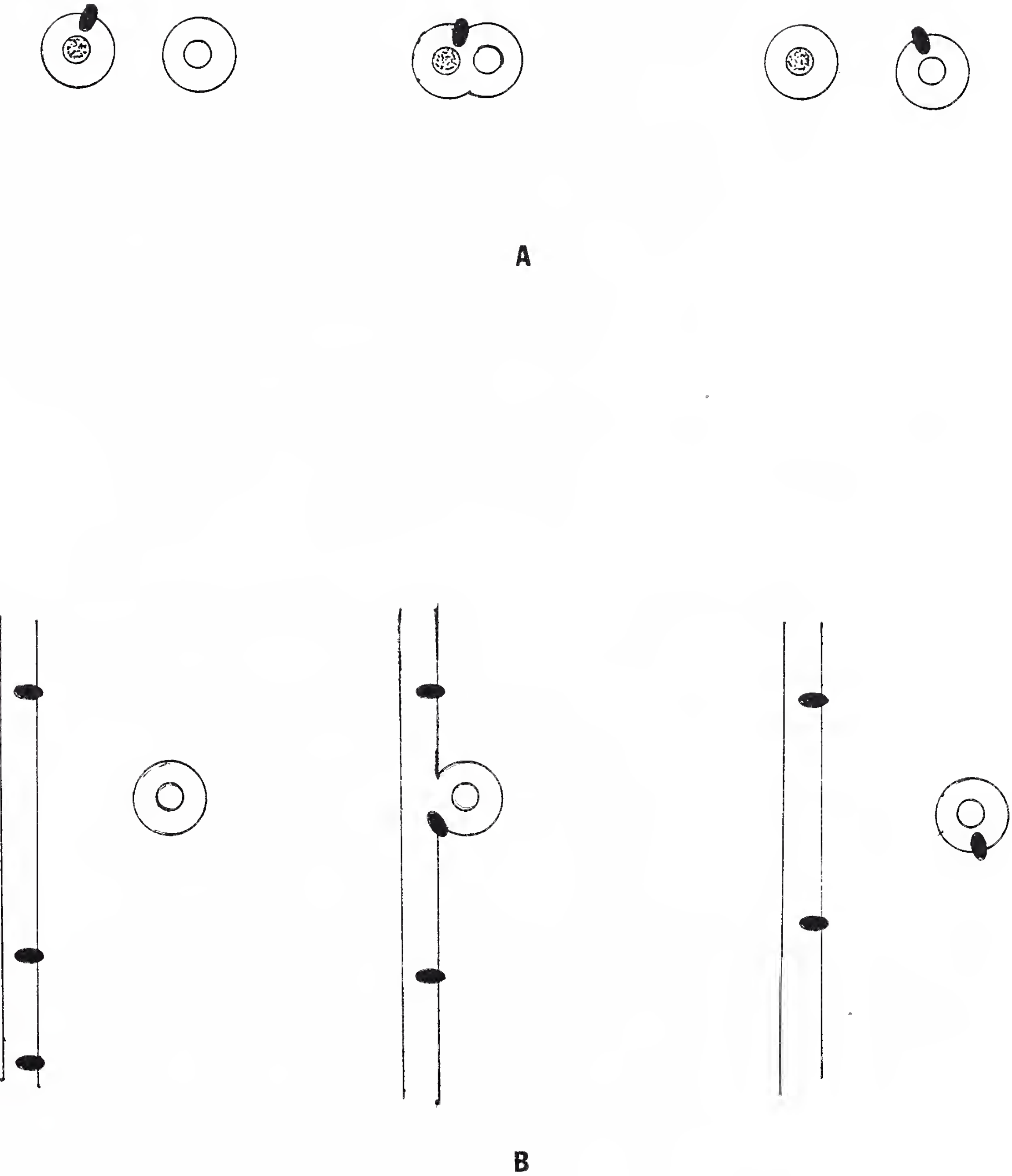


Figure 9.

molecular weight of 95 kilodaltons, similar to that of band 3, the red cell anion transport protein (Ott, et al., 1981).

If protein transfer from cells to vesicles takes place, the possibility of the reverse process, vesicle to cell transfer, occurring is raised. Bouma et al. (1977) attempted to demonstrate delivery of extracted proteins labelled with radioactive iodine back into cells using vesicles. Some of the label became associated with the cells, but this result could have been due to either protein transfer or to vesicle adsorption. Definitive proof of the ability of vesicles to transfer proteins to cells requires that a vesicle-associated intrinsic protein can be delivered to the cell membrane and express its functional activity in the cell membrane, and that the function of the transferred protein involves the alteration of intracellular properties. Of the proteins that are known or believed to be transferred from red cells to vesicles, only one is suitable for this purpose: band 3. The goal of the experiments described in this thesis is to determine if band 3 is one of the proteins extracted from erythrocytes, and, if so, to demonstrate that this protein can be reintroduced into cells.

Band 3 (nomenclature of Fairbanks, et al., 1971) is the major intrinsic protein of the red cell membrane, representing approximately 25% of the total membrane protein. When analyzed by SDS-polyacrylamide gel electrophoresis, it appears as a broad band spanning the 85 to 110 kilodalton molecular weight range (Fairbanks, et al., 1971). Band 3 appears to have several physiological functions, the most important of which is anion transport across the membrane.

The function of anion transport in the erythrocytes is to allow bicarbonate, formed from tissue carbon dioxide by red cell carbonic anhydrase, to distribute rapidly into the plasma. This prevents the accumulation of bicarbonate in the red cells and increases the ability of blood to transport carbon dioxide from peripheral tissues to the lungs. Transport occurs by one-for-one exchange of anions across the red cell membrane: in the tissue capillaries, intracellular bicarbonate is exchanged for extracellular chloride; in the lungs, the reverse process takes place. Band 3 also mediates unidirectional anion fluxes across the membrane. The rate constant for unidirectional transport, however, is several orders of magnitude smaller than that for exchange. Anion transport is driven by anion concentration gradients across the membrane and does not require metabolic energy (Cabantchik, _et al_., 1978). This system will transport a variety of anions, including halides, phosphate, sulfate, and some organic acids, although at different rates. The most rapid rate of transport occurs with chloride exchange, which has a half-life of about 50 milliseconds at physiological temperatures (Brahm, 1977), far less than the amount of time blood cells spend passing through tissue and lung capillary beds.

The study of anion transport in erythrocytes has been facilitated by the development of chemical probes that bind to band 3 and inhibit anion transport. The most widely-used of these probes have been isocyano derivatives of sulfonic acid, such as diisothiocyanostilbene disulfonic acid (DIDS). Useful characteristics of DIDS include (Cabantchik, _et al_., 1978):

1) very slow permeation limits its activity to the outer surface of the membrane

2) full, irreversible (covalent) inhibition of anion transport is produced by DIDS treatment under mild conditions
3) interference with no other transport or enzymatic properties of the red cell membrane
4) at maximal inhibitory concentrations, more than 90% of DIDS is bound to band 3

Measurement of labelled DIDS binding to red cells has allowed the number of band 3 molecules to be estimated at about 10^6 per cell (Ship, et al., 1977).

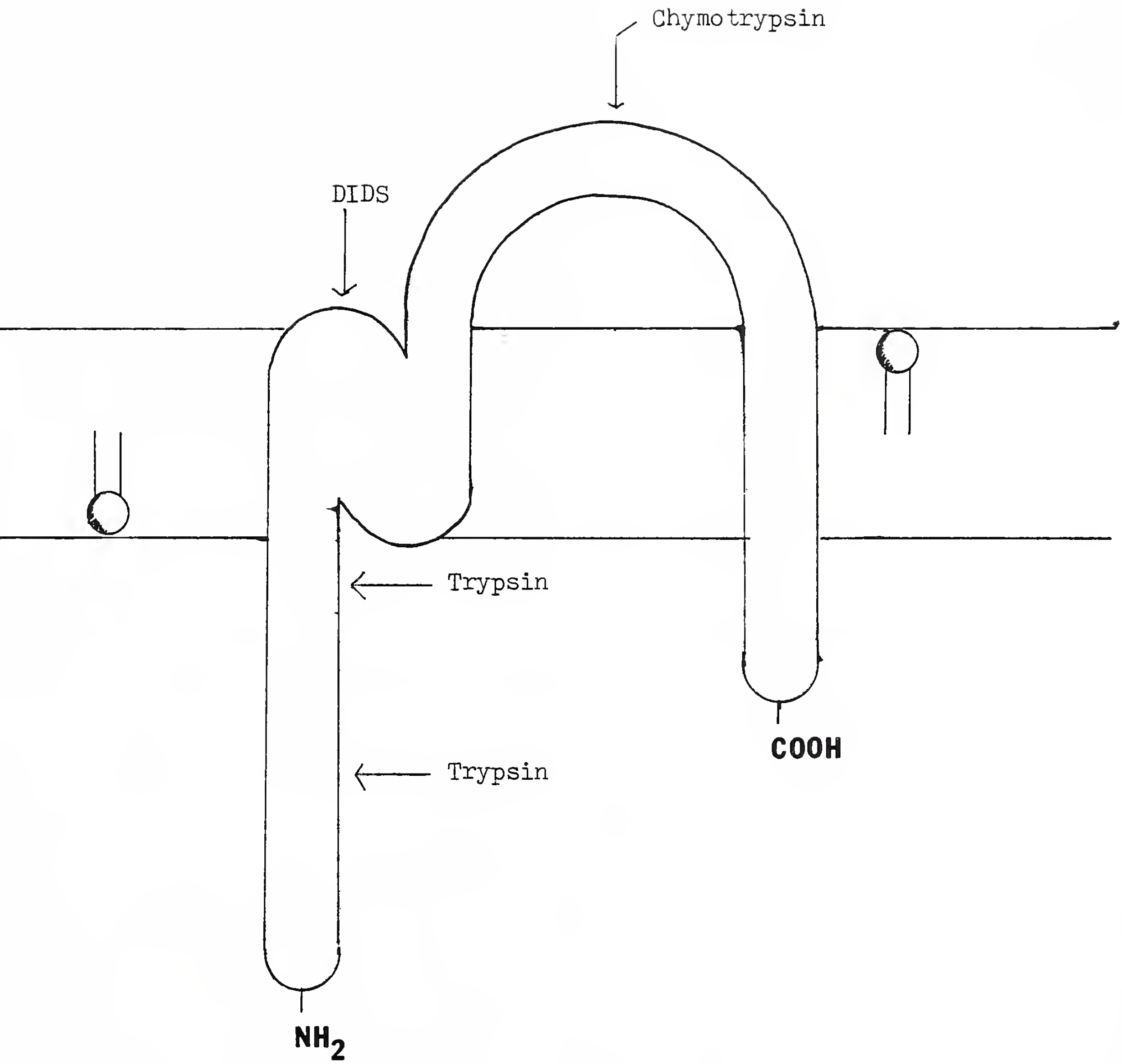
Other useful information about band 3 has been obtained by studying the products of its enzymatic digestion. Band 3 is a transmembrane protein that crosses the membrane at least twice: both the amino and carboxy terminals are on the cytoplasmic side of the membrane (Markowitz and Marchesi, 1981). Digestion with chymotrypsin at the external surface produces two membrane-bound segments with molecular weights of roughly 65 and 30 kilodaltons, which contain the amino and carboxy terminals, respectively (Drickamer, 1976). Treatment with trypsin at the inner membrane surface produces two large fragments. One is a membrane-bound segment with a molecular weight of about 55k; the other, a cytoplasmic segment whose molecular weight is roughly 40k. Smaller products of the digestion of the 40k segment are also produced (Jenkins and Tanner, 1977). The membrane-bound tryptic fragment, which contains the carboxy terminal, represents the segment of the protein that transports anions (Lepke and Passow, 1976). The cytoplasmic proteins, including: glyceraldehyde 3-P dehydrogenase (Yu and Steck, 1975), aldolase (Strapazon and Steck, 1977), hemoglobin (Sayare and Fikiet, 1981), and ankyrin (band 2.1) (Bennett and Stenbuck, 1979, 1980; Hargreaves, et al., 1980).

The DIDS binding site is on the 65k chymotryptic and 55k tryptic fragments (Drickamer, 1976). Vigorous chymotryptic digestion at both faces of the membrane produces a 15k transmembrane segment that can be cleaved by CNBr into three fragments of 7, 4, and 4 kilodaltons. The DIDS binding site is located on the middle 4k segment. Since the DIDS binding site is located near the outside face of the membrane, it appears that the 15k segment crosses the bilayer at least three times (Ramjeesingh, *et al.*, 1980). A schematic representation of the membrane orientation of band 3, based on the data presented above, is shown in Figure 10.

Band 3 contains five reactive sulfhydryl groups, all on the cytoplasmic side of the membrane (Rao, 1979). Three of these are located on the cytoplasmic tryptic fragment; the remainder, on the cytoplasmic carboxy terminal segment (Rao and Reithmeier, 1979). One of these sulfhydryl groups can be cross-linked to a sulfhydryl group on another band 3 molecule to form a dimer with a molecular weight of about 190k (Steck, 1972). Largely for this reason, band 3 was originally believed to be a dimer in the red cell membrane. More recent data, however, suggest that it may instead exist as a tetramer (Nigg and Cherry, 1980).

As noted above, one of the cytoplasmic proteins that binds to band 3 is ankyrin. Ankyrin is one of four extrinsic proteins (the others: spectrin, actin, band 4.1) that compose the red cell cytoskeleton, a filamentous matrix of proteins lining the inner surface of the red cell membrane. The cytoskeleton appears to determine the structural stability of the red cell (Lux, 1979). Band 3 may "anchor" the cytoskeleton to the cell membrane. Recent experiments suggest that band 3 does not move freely within the membrane, but that its lateral mobility is

Figure 10: The membrane conformation of band 3. The sites of cleavage by chymotrypsin (at the outer surface) and trypsin, and the DIDS binding site are shown.


Chymotrypsin
DIDS
Trypsin
Trypsin
COOH
NH2

determined by its interactions with the cytoskeleton (Nigg and Cherry, 1980; Golan and Veatch, 1980; Hargreaves, et al., 1980).

To summarize, band 3 is an interesting protein both for its physiologic function as a transport protein and for its putative role in contributing to the structural stability of the cell. As the most abundant membrane protein in the most easily studied human cell line, it provides a useful model for developing concepts and techniques that may be applicable to less accessible transport and structural proteins.

MATERIALS AND METHODS

Materials. Dimyristoylphosphatidylcholine (DMPC), bovine chymotrypsin, o-phenanthroline, and alpha-methyl mannoside were products of Sigma Chemical Company. $Na_3{}^{32}PO_4$ was purchased from New England Nuclear, $Na^{36}Cl$ from ICN, DIDS from Aldrich, eosin maleimide (eosin MA) from Molecular Probes, and Concanavalin A-Sepharose 4B (Con A-Sepharose) from Pharmacia. Human erythrocytes were obtained from adult volunteers and used within 36h of collection. Unless otherwise stated, experiments were conducted in phosphate-buffered saline (PBS) containing 150mM NaCl, 7.5mM Na_2HPO_4, 5mM KCl, 1mM $MgSO_4$, and 10mM glucose, adjusted to pH 7.4 with 1N HCl.

Methods

Preparation of phospholipid vesicles. DMPC (24mM) was suspended in buffer by mixing on a vortex stirrer. The lipid suspensions were sonicated for 30 min at 40 °C in a test tube (13 X 100 mm) suspended in a Heat Systems Ultrasonics model 9H bath sonicator filled to a depth of 3.6 cm with a solution of a few grams sodium dodecyl sulfate (SDS) in water. Vesicles prepared in this manner are monodisperse (250 to 300 Å in diameter) and unilamellar (van der Bosch and McConnell, personal communication). Thin layer chromatography showed that they contained less than 0.1% lyso-PC (Bouma, *et al.*, 1977).

Extraction of erythrocyte membrane proteins. Freshly-drawn human erythrocytes were separated from plasma by centrifugation, then washed three times by suspension in 4 volumes of 0.15M NaCl, followed by centrifugation. A final wash in buffer followed.

Aliquots of packed cells were warmed to 37 °C. Phospholipid vesicles were added in a volume equal to that of the cells. Incubation was carried out at 37 °C in an agitating water bath for 45 min unless otherwise noted. The cells were then pelleted by centrifugation at 3000 X g for 5 min, and the vesicle supernatant was again centrifuged at 3000 X g for 5 min to eliminate any contaminating cells. Incubation of vesicles with resealed ghosts were performed in the same manner.

Analysis of band 3 orientation and quaternary structure. Washed cells were suspended to a hematocrit of 50% in buffer. Chymotrypsin was added to yield a concentration of 200 μg/ml, and the suspension was agitated gently at 22 °C for 14h. The cells were pelleted and washed three times by resuspension in ten volumes of PBS. The cells were then incubated with vesicles as above, and cell and vesicle fractions were separated by centrifugation. Concurrently, undigested cells were incubated with vesicles. After separation from the cells, half of the vesicle fraction was treated with chymotrypsin (200 μg/ml) for 1h at 22 °C. Chymotrypsin was removed from the vesicle sample by passing the sample through a Sepharose 4B column (0.5 X 5 cm). Stroma were prepared from the normal and chymotrypsin-digested cells by lysis and repeated washings with 5mM Na_2HPO_4, pH 8.0. Stroma and vesicle samples were analyzed by SDS-polyacrylamide gel electrophoresis (Ames, 1974).

For Cu(II)-phenanthroline cross-linking experiments, red cell ghosts were prepared as described by Steck and Kant (1974). Unsealed ghosts were incubated for 30 min at 0 °C in ten volumes of 5mM Na_2HPO_4, pH 8.0, containing 50 μM $CuSO_4$ and 200 μM o-phenantroline. The membranes were pelleted, washed once, and resealed by suspension in 10 volumes of

5mM Na_2PO_4 plus 5 volumes of a solution containing 260mM KCl, 18mM NaCl, 0.875mM $MgCl_2$, 4mM Na_2HPO_4, 0.263mM $CaCl_2$, and 1mM Na_2ATP. After 5 min at 0 °C, the suspension was incubated 1h at 37 °C. The resealed ghosts were pelleted, washed once in PBS, and incubated with vesicles as above. Uncross-linked, resealed ghosts were prepared and incubated with vesicles also. Ghosts and vesicles were subjected to gel electrophoresis under reducing and non-reducing conditions.

Vesicle anion permeability. Anion flux into vesicles was measured by a modification of the method of Wolosin, et al. (1977). After incubation with cells, 0.5 ml of vesicles was added to 0.5 ml of PBS or PBS made 2mM in DIDS. Flux measurements were initiated by pipetting 3 μCi $Na_3{}^{32}PO_4$ into the rapidly stirred solution at 25 °C. At specified intervals, 100 μl aliquots were removed and anion flux quenched by the addition to an equal volume of 2mM DIDS in PBS at 0 °C. Aliquots (50 μl) of these samples were applied to 1.5 ml DEAE-Sephadex A-25 columns at 4 °C. Vesicles were eluted by addition of a precalibrated volume of PBS/2mM DIDS that eluted the vesicles but not free ${}^{32}PO_4{}^{-3}$. Eluted vesicles were collected directly into scintillation vials, dissolved in Aquasol, and analyzed by liquid scintillation counting.

Protein transfer from vesicles to cells. Band 3-vesicle complexes were isolated from the vesicle supernatant by a modification of the technique of Ross and McConnell (1977). Vesicle supernatants were applied to a Con A-Sepharose column (0.9 X 30 cm). The resin was washed with at least 3 column volumes of a buffer containing 0.2M NaCl, 10mM HEPES, 0.7mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$, and 0.5% NaN_3. The band 3-vesicle complexes were eluted with a similar buffer containing 0.1M NaCl and

0.1M alpha-methyl mannoside. The band 3-vesicle complexes were divided into two fractions. One fraction was treated with 0.7mM eosin MA for 1h in the dark at 22 °C to inhibit vesicle-bound band 3 (Nigg and Cherry, 1979). (Eosin MA was used as a covalent band 3 inhibitor because, unlike DIDS, it is monovalent and should not crosslink and aggregate band 3-vesicle complexes). Unbound eosin MA was removed by passing the sample through a PD-10 column. Washed red cells were incubated with an equal volume of 0.7mM eosin MA in PBS for 3h in the dark at 22 °C, and isolated by centrifugation and washings in PBS. Samples of eosin-inhibited cells were incubated with a) isolated band 3-vesicle complexes, b) eosin-inhibited band 3-vesicle complexes, and c) PBS, each vesicle sample having a phospholipid concentration of 0.35mM. Control samples of untreated red cells were incubated simultaneously, suspended in either PBS or PBS containing pure DMPC vesicles in the same concentration as in the experimental samples. All cell samples were pelleted, washed three times in PBS, and used immediately for chloride flux measurements.

Measurements of chloride flux into cells. Red cells (0.5 ml) were suspended in rapidly stirred PBS (4.5 ml) on ice. $Na^{36}Cl$ (1.9 μCi) was added. At specified intervals, 0.5 ml aliquots of the suspension were pipetted into 0.2mM DIDS in PBS (0.9 ml). The cells were pelleted and washed by resuspension in 1.0 ml 0.2mM DIDS in PBS. Aliquots (20 μl) of packed cells were lysed in 0.5 ml water, dissolved in 10 ml Aquasol, and analyzed by liquid scintillation counting.

RESULTS

Extraction of band 3 from erythrocytes using DMPC vesicles

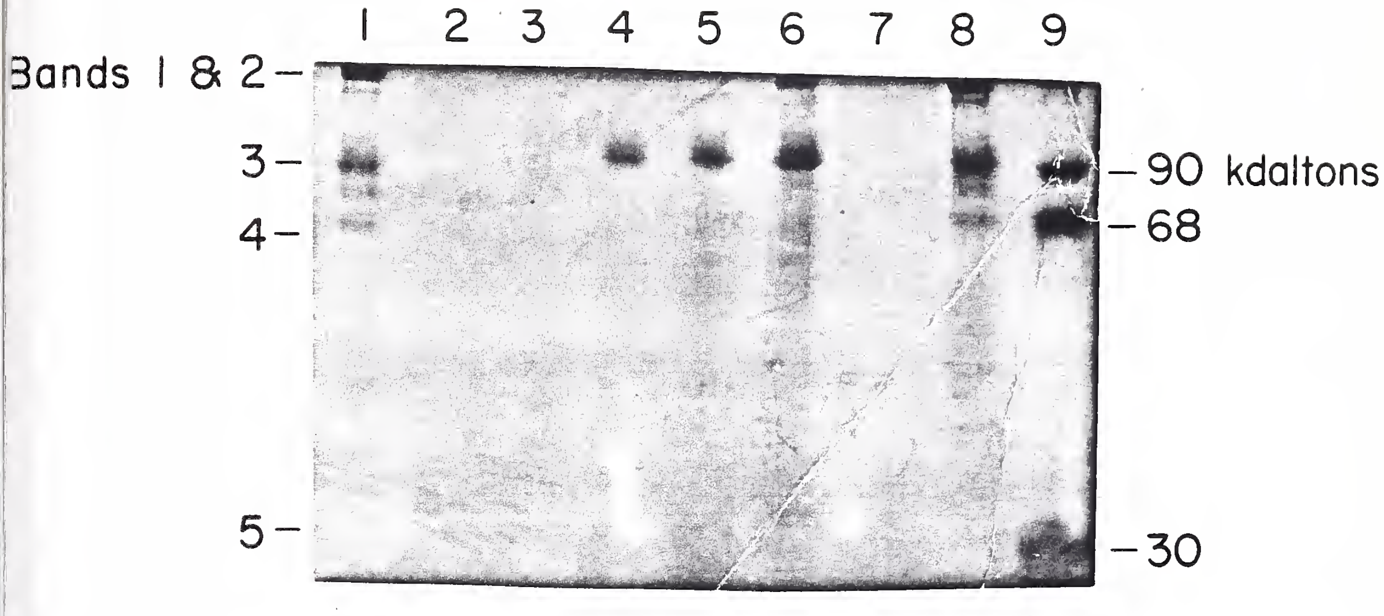
Figures 11 through 13 are photographs of SDS-polyacrylamide gels performed with vesicles and washed stroma.

Figure 11 demonstrates the appearance of extracted cell proteins into the vesicles. Lane 1 contains washed stroma and demonstrates the major protein bands of the membrane. Lanes 2 through 6 reveal the proteins bound to vesicles after 0, 15, 30, 45, and 60 minutes of incubation with cells at 37 °C. The most prominent vesicle-associated protein co-migrates with band 3 and shows the same diffuse distribution around 95k daltons. Bands 1 and 2 do not appear in the vesicle fraction until significant cell lysis has occurred.

The effects of chymotrypsin treatment of the 95k protein are seen in Figure 12. Digestion of cell membranes or vesicle-protein complexes results in the disappearance of band 3 and the appearance of a band at about 65k daltons (lanes 4 and 3, respectively). If vesicles are incubated with chymotrypsin-treated cells, the same 65k dalton band appears in the vesicles (lane 6).

The gel in Figure 13 contains proteins of red cell stroma cross-linked by Cu(II)-phenanthroline and analyzed by electrophoresis in the absence (lane 4) and presence (lane 9) of β-mercaptoethanol. Band 3 is replaced by higher molecular weight bands unless the sample is reduced. Vessicles incubated with cross-linked, resealed ghosts contain no band 3 (lane 5). When this sample is reduced (lanes 10 and 11), band 3 appears and higher molecular weight bands disappear. Vesicle-bound band 3

Figure 11: Sodium dodecylsulfate polyacrylamide gel (10%) of erythrocyte membranes (lanes 1 and 8) and phospholipid vesicles incubated with cells for 0 (lane 2), 15 (lane 3), 30 (lane 4), 45 (lane 5), and 60 (lane 6) minutes at 37 °C. Prior to electrophoresis, vesicles were separated from unbound protein (lane 7) by passage through Sepharose 4B. Bands 1 and 2 do not appear in the vesicles until significant hemolysis has occurred (lane 6).


1
2
3
4
5
6
7
8
9
Bands 1 & 2
3
4
5
90 kdaltons
68
30

1. Ghosts
2. Vesicles, t = 0 minutes
3. Vesicles, t = 15
4. Vesicles, t = 30
5. Vesicles, t = 45
6. Vesicles, t = 60
7. Soluble fraction, t = 30
8. Ghosts
9. Standard

Figure 12: Polyacrylamide gel of normal and chymotrypsin-treated band 3 in cells and vesicles. Standards (lanes 1 and 6), normal ghost membranes (lane 2), vesicle extract of normal cells (lane 3), chymotrypsin-treated extract of normal cells (lane 4), chymotrypsin-treated ghosts (lane 5), and vesicle extract of chymotrypsin-treated cells (land 6).

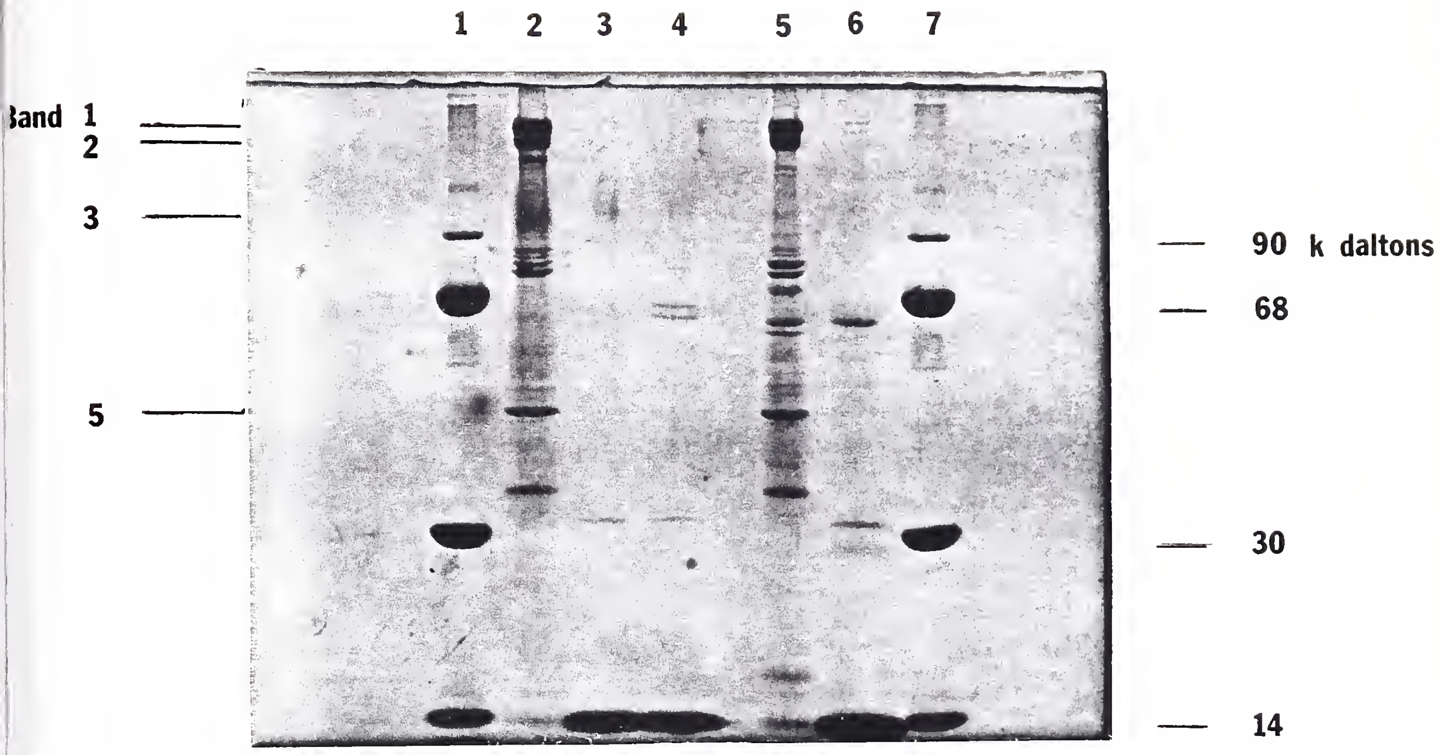
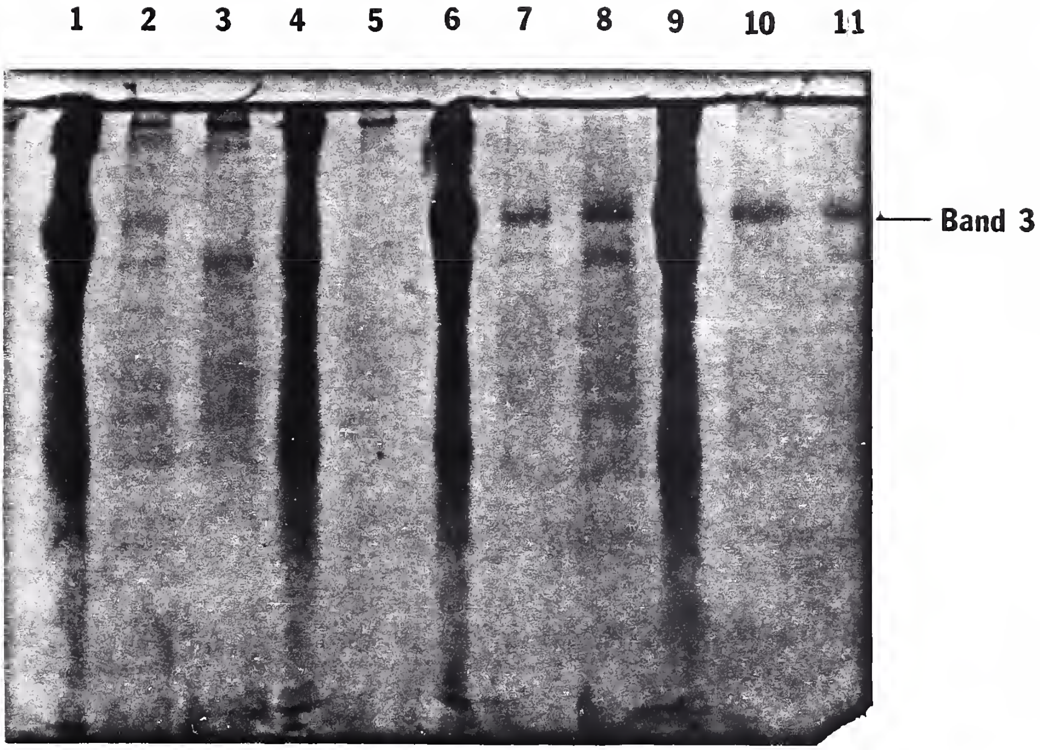

1
2
3
4
5
6
7
Band 1
2
3
5
90 k daltons
68
30
14

Figure 13: Polyacrylamide gel of normal and Cu(II)-phenanthroline cross-linked band 3 in cells and vesicles. Normal ghosts (lane 1), vesicles incubated with normal ghosts (lane 2), Cu(II)-phenanthroline treated vesicle extract of normal ghosts (lane 3), Cu(II)-phenanthroline treated ghosts (lane 4), and vesicle extract of Cu(II)-phenanthroline treated ghosts (lane 5). Lanes 6-10 show the same samples in the same order, subjected to electrophoresis in the presence of β-mercaptoethanol.


1
2
3
4
5
6
7
8
9
10
11
Band 3

could also be cross-linked (lane 3); again, this was reversed by treatment with β-mercaptoethanol (lane 8).

Anion permeability of band 3-vesicle complexes

After incubation with erythrocytes, vesicles exhibited anion uptake (Figure 14). Anion uptake was not observed in the presence of DIDS or in vesicles not exposed to cells. These results indicate that band 3 retains the ability to transport anion across the vesicle membrane. Moreover, since anion uptake into the vesicles is quantitatively inhibited by DIDS, the protein must be in its native orientation.

Transfer of band 3 from vesicles to cells

Figure 15 presents the protocol for examining transfer of band 3 from vesicles to cells. Erythrocytes in which anion transport had been irreversibly inhibited with eosin MA were incubated with: a) band 3-vesicle complexes purified on a Con A-Sepharose column, b) purified band 3-vesicle complexes that had been treated with eosin MA, c) PBS. After the incubations, the chloride uptake of the cells was measured. These results are shown in Figure 16.

Cells treated with eosin MA exhibited a rate of chloride uptake that was 1.3% of uptake into treated cells, i.e., uptake was inhibited almost 99% by treatment with eosin MA. Incubation of inhibited cells with eosin-treated vesicles produced no increase in chloride uptake. Incubation of inhibited cells with functional band 3-vesicle complexes, however, produced a small, but significant increase in chloride uptake ($t < 0.01$) (Calculation performed with Student's t-test using triplicate determinations of chloride uptake at 15 min). In all cases, chloride

Figure 14: ^{32}P-phosphate flux into protein-vesicle complexes at 25 °C in the absence (○) and presence (●) of DIDS.

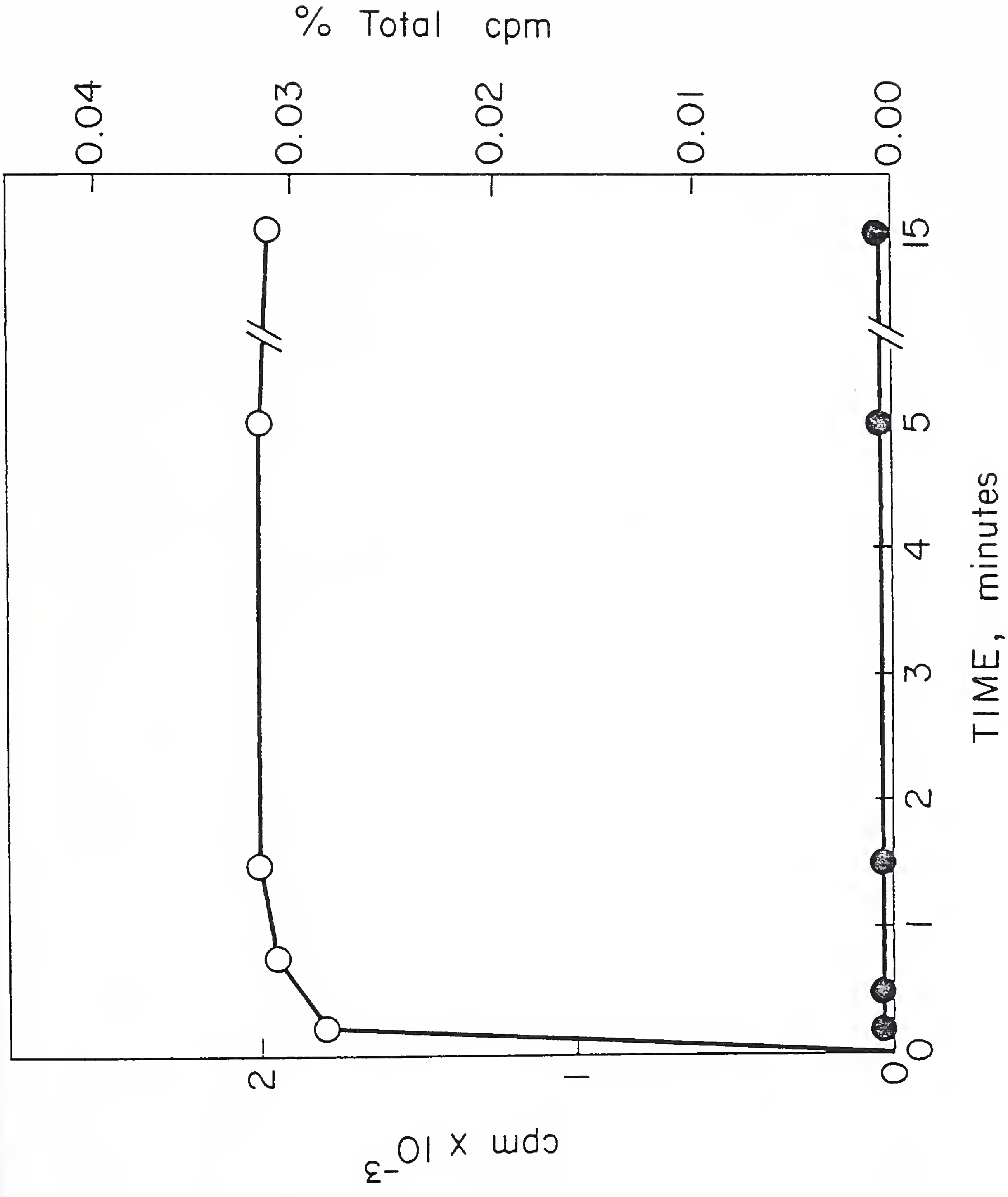

% Total cpm
0.04
0.03
0.02
0.01
0.00
cpm x 10^{-3}
2
1
0
TIME, minutes
0
1
2
3
4
5
15

Figure 15: Protocol for delivery of band 3 into red cells whose anion transport has been irreversibly inhibited with eosin MA.

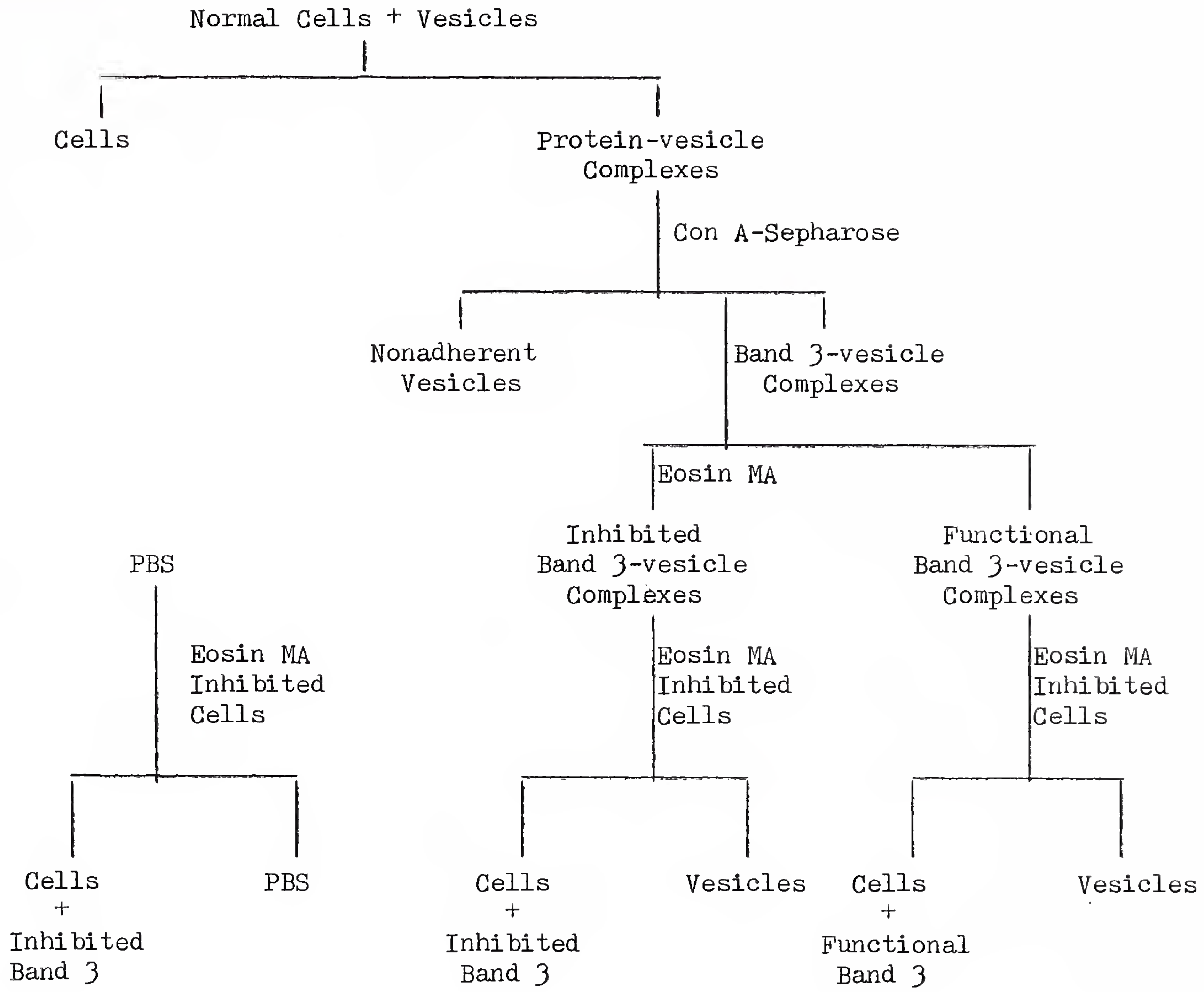
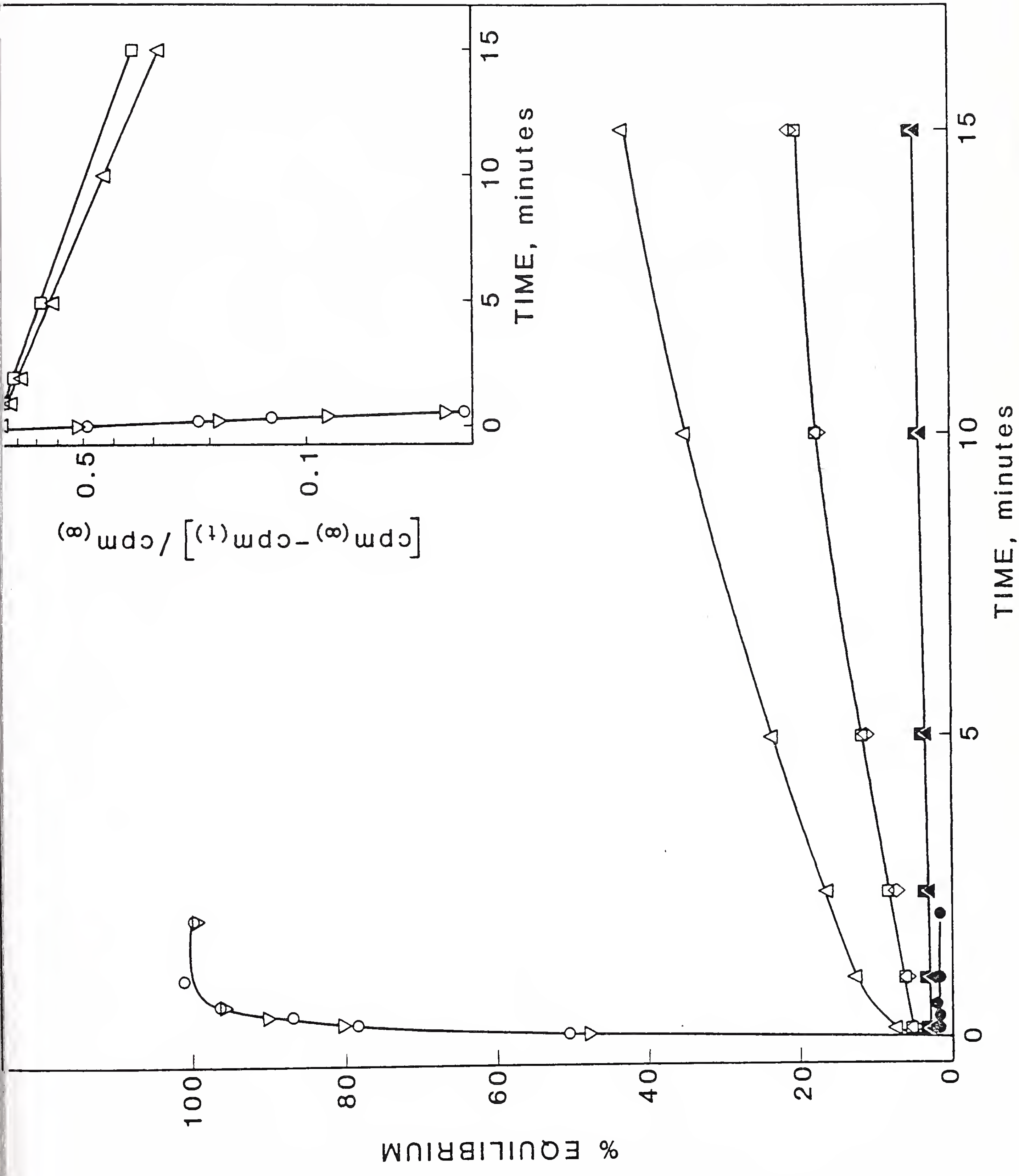

Normal Cells + Vesicles
Cells
Protein-vesicle Complexes
Con A-Sepharose
Nonadherent Vesicles
Band 3-vesicle Complexes
Eosin MA
Inhibited Band 3-vesicle Complexes
Functional Band 3-vesicle Complexes
PBS
Eosin MA Inhibited Cells
Eosin MA Inhibited Cells
Eosin MA Inhibited Cells
Cells + Inhibited Band 3
PBS
Cells + Inhibited Band 3
Vesicles
Cells + Functional Band 3
Vesicles

Figure 16: Chloride uptake into cells at 0 °C. Normal erythrocytes (○), after incubation with DMPC vesicles (▽); eosin MA inhibited cells after incubation with functional band 3-vesicle complexes (△), eosin MA inhibited band 3-vesicle complexes (□), or PBS (◇). Filled symbols show chloride uptake into the same samples in the presence of 0.2mM DIDS.


% EQUILIBRIUM
0
20
40
60
80
100
TIME, minutes
0
5
10
15
[cpm(∞)-cpm(t)]/cpm(∞)
0.5
0.1
TIME, minutes
0
5
10
15

uptake was blocked quantitatively ($>$99.98%) by the presence of 0.2mM DIDS during uptake measurements. All observed anion uptake, therefore, was mediated by band 3.

The fraction of cells in the samples containing one or more functional anion transport proteins can be estimated by fitting plots of ln $(\mathrm{cpm}(\infty) - \mathrm{cpm}(t)/\mathrm{cpm}(\infty))$ versus time for arbitrary values of $\mathrm{cpm}(\infty)$. For the inhibited cells treated with functional or inhibited band 3-vesicle complexes, the best fits for this function were obtained when $\mathrm{cpm}(\infty)$ corresponded to 55% and 24% of the cells, respectively. Therefore, when eosin-inhibited cells were treated with functional band 3-vesicle complexes, at least 31% of the cells acquired band 3 from the vesicles.

DISCUSSION

The experiments described in the results section were performed before the heterogeneity of vesicle-protein complexes produced by incubation of erythrocytes with DMPC vesicles was appreciated. The results shown in Figures 11 through 14 were obtained with vesicle samples that contained both transfer vesicles and vesicle buds. These experiments have recently been repeated with pure transfer vesicles and vesicle buds, and identical results were obtained with both samples (Newton and Huestis, personal communication).

Analysis of the purified band 3-vesicle complexes eluted from Con A-Sepharose columns has revealed that these vesicles are pure transfer vesicles (Newton and Huestis, personal communication). Therefore, the experiments examining vesicle-to-cell protein transfer were performed with transfer vesicles, not a mixture of transfer vesicles and vesicle buds.

These experiments demonstrate the utility of using a transport protein to demonstrate vesicle-to-cell protein transfer. The number of band 3 copies transferred from vesicles to cells in these experiments is small relative to the total number of copies in normal cells: incubation of functional band 3-vesicle complexes with inhibited cells produced only a 15% increase in the rate of chloride flux (from 1.3% to 1.5% of normal). Since the percentage of cells with at least one functional anion transport protein was increased 130% from 24% to 55%, however, the increase in chloride flux was statistically significant. Therefore, this method of examining protein transfer to cells is extremely sensitive. These results could not be due to adsorption of

band 3-vesicle complexes to cells. If the vesicles were merely adsorbed to the cells, cells treated with functional band 3-vesicle complexes would show a much smaller increase in anion uptake because the enclosed volume of the vesicles is more than two orders of magnitude less than that of the cells.

These results could also be produced by fusion of band 3-vesicle complexes with cells. This possibility is unlikely, however, since the vesicles used in the vesicle-to-cell transfer experiments were transfer vesicles, which do not contain PS. It is possible that some other lipid or protein component in the transfer vesicles can induce cell-vesicle fusion. If this were true, though, vesicle buds and transfer vesicles would be expected to fuse with each other, which they do not. These observations do not exclude the possibility of cell-vesicle fusion, but current data is most consistent with an intermembrane transfer mechanism.

Another system in which protein transfer between membranes has been described is the transfer of cytochrome b_5 between vesicles (Roseman, et al., 1977; Enoch, et al., 1977). This phenomenon has been shown to occur by the protein diffusing through solution between membranes, rather than by membrane-membrane collisions (Leto, et al., 1980). Cytochrome b_5 is much less hydrophobic than band 3 and is not a transmembrane protein. Considering the extremely hydrophobic nature of band 3 segments associated with the cell membrane (Steck, 1978), it is most unlikely that transfer of band 3 between membranes could occur by the protein diffusing through an aqueous solution.

It may seem paradoxical that band 3, which is thought to be bound to the red cell cytoskeleton, could be so easily removed from the cell

by budding and transfer. The average number of band 3 copies removed from each cell can be crudely estimated based on measurements of the relative areas of the band 3 and AChE peaks on densitometer scans of polyacrylamide gels performed on vesicle extracts; determination of the percentage of AChE activity removed from cells by vesicles; and estimation of the number of AChE copies to be 10^4 per cell (Sihotang, 1974). Using this method, the number of band 3 copies removed per cell is 10^5, or 10% of the total. Only about 15-20% of band 3 appears to be directly bound to ankyrin (Nigg and Cherry, 1980; Hargreaves, et al., 1980). If band 3 is assumed to be a tetramer, then approximately 60-80% would be anchored to the cytoskeleton. Therefore, the observed amount of band 3 extraction into vesicles could occur without requiring the dissociation of any band 3 from the cytoskeleton. Cytoskeletal binding may, in fact, represent the reason that removal of band 3 is inefficient relative to AChE removal.

Much still needs to be learned about intermembrane protein transfer, particularly the factors that determine the specificity and kinetics of the process. Unfortunately, the study of protein transfer from erythrocyte membranes to vesicles is complicated by the simultaneous occurrence of membrane budding. For reliable information to be gained about cell-to-vesicle transfer, a system must be found in which budding does not take place.

The occurrence of intermembrane protein transfer in vitro raises the question of whether this phenomenon also occurs in any biological systems. No such process has yet been described, but intermembrane protein transfer is an attractive mechanism for membrane assembly.

In the laboratory, protein transfer could become a powerful tool in studying membrane physiology in normal and diseased cells. Vesicle-to-cell transfer is a uniquely "non-invasive" technique for altering the

properties of a cell: it could be used to introduce a foreign protein into a cell, or a normal protein into a cell in which that protein is defective. Hopefully, the techniques employed in this thesis will be useful in learning more about intermembrane protein transfer, and in applying this interesting phenomenon to the study of other systems.

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